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ZENECA Limited
15 Stanhope Gate
LONDON W1Y 6LN, Great Britain
Patents ADP number (if you know it)
254007002

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

DEVICE

5. Name of your agent (if you have one)

DENERLEY, Paul Millington
"Address for service" in the United Kingdom
to which all correspondence should be sent
(including the postcode)
Intellectual Property Department
ZENECA Pharmaceuticals
Mereside, Alderley Park
Macclesfield, Cheshire, SK10 4TG, Great Britain
Patents ADP number (if you know it) 1030618002

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Priority application number
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Date of filing
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Number of earlier application

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Description 11

Claim(s) -

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I/We request the grant of a patent on the basis of this application.

Signature

Lynda M Slack

Date

13 Oct 1998

11. 12. Name and daytime telephone number of
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Lynda M Slack
01625 516173

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DEVICE

Disclosed is an assay device which is able to detect molecules which inhibit the binding of a ligand to a receptor using extremely small quantities of ligand and receptor sample. Such devices are useful in the discovery of molecules which may modulate the activity of biologically important target molecules.

Currently the pace of change in techniques and tools for discovery of biologically active molecules is increasing with the ability of combinatorial chemistry and multi-parallel synthesis (MPS) to rapidly provide large numbers of diverse molecules to test for biological activity. In addition the mapping and sequencing of the genomes of many plants, animals and parasites, including the human genome, is already providing a growing number of new targets which may be used in biological tests. In the future it is to be expected that the number of biological targets is to grow even further. It is estimated that in the last 100 years of research only 400 human drug targets have been discovered whilst the human genome project when completed at the scheduled time of 2005 will have sequenced at least 100,000 genes, many of which will code for important biological targets for drug therapy.

However, synthetic techniques such as MPS and combinatorial chemistry provide relatively small sample sizes, for example in the microgram range. The limited sample sizes currently produced are not large enough to supply more than a few biological tests before the supply is exhausted. Therefore, resynthesis is required in order to restock the chemical library.

Currently there is an enormous range of *in vitro* assay techniques used for biological tests. Generally an *in vitro* biological test involves exposing the isolated biological target to the compound under investigation and measuring interference with the normal binding of the biological target to its ligand. Such tests are typically run on standard 96 well plates and require a minimum sample size of biological target of around 0.1ml and a minimum amount of a sample size of compound of around 1 µg.

As well as supplies of the test compound running out, supplies of biological target molecules also may be quickly depleted. It is an expensive and tedious task to have to express, isolate and purify the receptor from the biological source.

5 Therefore, there is a need to find simple, sensitive, high-throughput approaches to the identification of compounds inhibiting ligands binding to biologically important target molecules for use in the pharmaceutical and agrochemical industry. Such a system should use small sample sizes and be amenable to operation by a machine. In particular, miniaturised approaches operating on the picolitre / nanolitre / microlitre scale are particularly desirable,
10 because of the large cost savings and potential for very high throughput. So far there are very few approaches that will work satisfactorily at this scale. One such approach, fluorescence correlation spectroscopy, which is based on differences in diffusion constant measured by fluorescence in femtolitre interrogation volume is inherently slow because it measures only small numbers of molecules.

15 Microfabrication techniques are general known in the art using tools developed by the semiconductor industry to miniaturise electronics, it is possible to fabricate intricate fluid systems with channel sizes as small as a micron. These devices can be mass-produced inexpensively and are expected to soon be in widespread use for simple analytical tests. See,
20 e.g., Ramsey, J.M. et al. (1995), "Microfabricated chemical measurement Systems," Nature Medicine 1:1093-1096; and Harrison, D.J. et al (1993), "Micromachining a miniaturized capillary electrophoresis-based chemical analysis system on a chip," Science 261:895-897.

25 Miniaturisation of laboratory techniques is not a simple matter of reducing their size. At small scales different effects become important, rendering some processes inefficient and others useless. It is difficult to replicate smaller versions of some devices because of material or process limitations. For these reasons it is necessary to develop new methods for performing common laboratory tasks on the microscale.

30 Devices made by micromachining planar substrates have been made and used for chemical separation, analysis, and sensing. See, e.g., Manz, A. et al. (1994), "Electroosmotic pumping

and electrophoretic separations for miniaturized chemical analysis system," J. Micromech. Microeng. 4:257-265.

We have found that microfabricated devices can be used in studying the binding of 5 compounds to biological targets, for instance a binding assay. Such microfabricated assays are based on differences in diffusion between free ligand and ligand bound to the biological target, the "receptor".

In its simplest form the microfabricated assay has a liquid containing diffusion chamber 10 within which is a limited area, the "diffusion region", exposed to a receptor, a ligand for the receptor and a test compound, the "mixture". If the test compound inhibits the ligand receptor interaction then the ligand is free to diffuse to areas of the diffusion chamber outside the diffusion region. Diffusion of free ligand is much more rapid than for ligand which is bound to the receptor since there is a difference in molecular weight between the ligand and the 15 ligand receptor complex and, therefore, a difference in the speed of diffusion. In general the receptor will be of a higher molecular weight than the ligand.

Since the cost of microfabricating such devices is low many of these diffusion chambers may be produced in parallel in a single use disposable array for simultaneous analysis of many test 20 compounds. Alternatively such devices may be reused.

In an alternative, and preferred, feature liquid is streamed through the diffusion chamber, which forms a microfabricated conduit, allowing for sequential introduction of different test compounds into the same diffusion chamber, and thereby continual serial analysis in the same 25 diffusion chamber is achievable. In addition a number of these microfabricated conduits may be placed in parallel and thus a very high throughput of test samples may be achieved. With this feature of the invention the size and dimension of the microfabricated conduit is such that laminar flow of the liquid is maintained, at least between the points where the mixture is introduced and the detection area. In such a device the mixture is introduced into the 30 diffusion region of the microfabricated conduit which does cross the entire cross section of the laminar flow of liquid. As the laminar flow passes through the microfabricated conduit any

free ligand or test compound will diffuse out of the diffusion area and across the width of laminar flow into parts which do not yet contain any free ligand or test compound.

Detection of the presence or absence of free ligand or test compound inside or outside the 5 diffusion area may be by external detection means for detecting changes in an indicator substance present in the liquid of the diffusion chamber, by the use of adsorption spectroscopy or fluorescence, by immunological means, by electrical means, by radioactive means or by the use of any convenient detection system. The laminar flow may be separated at the detection area and the separated laminar flow liquid may then be sampled for the presence or absence of 10 unbound ligand or test compound. The concentration of any substance outside the diffusion area may also be measured. Alternatively, simpler detection systems may be employed which monitors the level of fluorescence at a single point (or a few points).

Accordingly we present as a first feature of the invention a microfabricated binding assay 15 device comprising:

(1) a microfabricated diffusion chamber,
(2) a diffusion region within the microfabricated diffusion chamber, and
(3) at least one inlet for introducing liquid into the microfabricated diffusion chamber and for 20 introducing into the diffusion region a test compound, a receptor and a ligand for the receptor, such that in use the binding of the ligand to the receptor may be interrupted by the test compound and the ability of the test compound to interrupt the binding of the receptor and the ligand is determined by reference to the diffusion of the test compound, the receptor or the ligand out of the diffusion region.

25

We present as a further feature of the invention a method for determining the ability of a test compound to interfere with the binding of a ligand to a receptor in a microfabricated device which method comprises:

30 (1) introducing the test compound, the receptor and the ligand into a diffusion region of a microfabricated diffusion chamber,

(2) introducing liquid into parts of the microfabricated diffusion chamber not occupied by the diffusion region, and

(3) detecting the diffusion of the test compound, the receptor or the ligand out of the diffusion region.

It will be understood that in the above method steps (1) and (2) may be reversed, or occur simultaneously. Preferably the diffusion of the ligand in step (3) is detected, and preferably the ligand is labelled.

10

The diffusion chamber may be any convenient shape, such that the liquid and the mixture in the diffusion region are brought together without mixing. The maximum dimension of the diffusion chamber is up to 500 μm , preferably up to 200 μm . The minimum dimension is at least 2 μm , preferably at least 5 μm .

15

The diffusion region is an area of the diffusion chamber sufficiently large enough that differential diffusion of bound from unbound ligand, or test compound, can be determined.

There may also be a separate inlet for the introduction of liquid into the diffusion chamber, 20 and a separate inlet for introducing the mixture, or different inlets for introducing each component of the mixture, or combinations thereof, into the diffusion region.

Optionally one of the components may be introduced into the whole of diffusion chamber, and thereby also the diffusion region, by addition with the liquid and the remaining two components of the mixture added just to the diffusion region.

25

In addition to an inlet there may also be an outlet for removing the liquid and mixture from the diffusion chamber to waste.

We present as a further feature of the invention a microfabricated binding assay device 30 comprising;

(1) an internal surface defining a microfabricated conduit,
(2) a diffusion region within the microfabricated conduit smaller than the area of cross section
of the microfabricated conduit,
(3) the microfabricated conduit having at least one inlet for introducing liquid in laminar flow
5 and introducing into the diffusion region a test compound, a receptor and a ligand for the
receptor, and
(4) an outlet for exiting liquid in laminar flow, such that in use the binding of the ligand to the
receptor may be interrupted by the test compound and the ability of the test compound to
interrupt the binding of the receptor and the ligand is determined by reference to the diffusion
10 of the test compound, the receptor or the ligand across the laminar flow and out of the
diffusion region.

We present as a further feature of the invention a method for determining the ability of a test
compound to interfere with the binding of a ligand to a receptor in a microfabricated device
15 which method comprises:

(1) introducing the test compound, the receptor and the ligand into a diffusion region of an
internal surface defining a microfabricated conduit, the diffusion region within the
microfabricated conduit being smaller than the area of cross section of the microfabricated
20 conduit,

(2) introducing liquid into the remaining cross section of the microfabricated conduit not
occupied by the diffusion area,
25 (3) ensuring that the mixture in the diffusion region and the liquid flow through the
microfabricated conduit in laminar flow, and

(4) detecting the diffusion of the test compound, the receptor or the ligand across the laminar
flow and out of the diffusion region.

30

It will be understood that in the above method steps (1) and (2) may be reversed, or occur

simultaneously. Preferably the diffusion of the ligand in step (3) is detected, and preferably the ligand is labelled.

The term "laminar flow" means stable flow of the liquid through the microfabricated conduit, 5 there being no areas of turbulence. Therefore the presence of compound or ligand unbound to the receptor in the flow width above the point of introduction of the mixture is entirely due to diffusion and no other effect.

Laminar flow occurs typically in a microfabricated conduit having a depth of no more than 10 100 μ m, preferably no more than 50 μ m. The depth, or width, of the microfabricated conduit is at least 2 μ m, preferably at least 5 μ m. Preferably the microfabricated conduit has a constant width with a smooth internal surface of the diffusion chamber.

In this disclosure, the term "ligand" refers to any substances of biological or chemical origin, 15 which bind to the receptor to form a complex which has a significantly different diffusion rate to free ligand. Preferably the ligand is labelled. Preferred labels include fluorescent labels and electrochemical luminescent labels.

In this disclosure, the term "receptor" refers to any substances, preferably of biological origin, 20 which is desired to be screened in a binding test, and which binds to the ligand or molecule to form a complex which has a significantly different diffusion rate to free ligand. Examples include enzymes, biological molecules, transcription factors, cell signalling molecules, DNA and RNA. The receptor may be a soluble molecule, e.g. a protein, or an insoluble species, for example a membrane fragment. Optionally the receptor may be a molecule on the surface of a 25 cell. Optionally the receptor may be an antibody, in which case the device may be used to carry out an immunoassay. In certain circumstances it may be advantageous to increase the molecular weight of the receptor by conjugation to a macromolecule, particles, liposomes, vesicles and the like. Such conjugation may be either covalent or non-covalent, for example mediated by specific binding pairs such as biotin and streptavidin.

In this disclosure, the term "compound" refers to any substance of biological or chemical origin.

In this disclosure, the term "significant different diffusion" means diffusion rate of sufficient difference to enable differentiation of diffusion between two molecules to be detected or measured across the point of introduction of the molecules and the detection area. By significant we mean preferably at least 10-fold, more preferably at least 100-fold difference in molecular weight.

10 In this disclosure, the term "microfabricated" includes devices capable of being fabricated on silicon wafers readily available to those practising the art of silicon microfabrication and having the feature sizes and geometries producible by such methods as LIGA, thermoplastic micropattern transfer, resin based microcasting, micromolding in capillaries (MIMIC), wet isotropic and anisotropic etching, laser assisted chemical etching (LACE), and reactive ion etching (RIE), or other techniques known within the art of microfabrication. In the case of silicon microfabrication, larger wafers will accommodate a plurality of the devices of this invention in a plurality of configurations. A few standard wafer sizes are 3" (7.5cm), 4"(10cm), 6"(15cm), and 8"(20cm). Application of the principles presented herein using new and emerging microfabrication methods is within the scope and intent of the invention.

15 20 Microfabricated devices are created through innovative combinations of three essential manufacturing processes: (1) photolithography, the optical process of creating microscopic patterns (2) etching, the process that removes substrate material and (3) deposition, the process whereby materials with a specific function can be coated onto to surface of the substrate.

25

In this disclosure, the term "liquid" means either an aqueous or non-aqueous liquid, preferably aqueous. In addition the liquid may be buffered and contain any number of molecules essential for the maintained function of the receptor.

30 It is preferable that the relative concentrations of ligand and receptor are such that if the test compound were absent little free unbound ligand would be present and, therefore, the

presence of any ligand outside the diffusion area is due only to the displacement of bound ligand by the test compound.

Alternatively the device and method of the invention may be used for 2 component assays 5 where a ligand is not employed. Here the test compounds are labelled and the extent of binding to the receptor is determined by the amount of slower diffusing bound compound and/or the amount of faster diffusing free compound.

The inlet of the device may have a single channel or a number of channels meeting prior to or 10 at the point of introduction of the mixture into the liquid. There also may be a channel for each component of the mixture. Various combinations of channels can be envisaged and in what order they meet before entry into the liquid at the inlet. A compound channel may preferably be mixed with the receptor channel prior to the addition of the ligand. In this way ligand will not slowly diffuse across the laminar flow if the compound is inactive.

15 Alternatively the receptor channel and ligand channel may meet before the compound channel, or receptor compound complex may be preformed prior to input into the device. In a further alternative the receptor, ligand and compound are mixed prior to input into the device in an "incubation area". This has the advantage that the period for binding of the compound to receptor may be controlled. An incubation area may simply be the length of stretch of 20 channel that is between the points where the components of the mixture are introduced and entrance into the diffusion region.

The inlet may operate in a pulsed mode, i.e. different compounds are sequentially fed into the system, separated by a buffer "plug" so that the system may operate in a continuous manner.

25 Designs to achieve this will depend on the way that compounds are made available to the system and will be apparent to the microtechnologist of average skill. The compounds entering the device may be from a separation system, such as a chromatography column, attached to the microfabricated device. Thus the device becomes useful for identifying active compounds from a mixture.

Further variations on the basic concept will be apparent to the skilled reader and are incorporated in this invention.

The device described here will be capable of high throughput, e.g. tens of determinations per 5 minute. Since the device may be fabricated at a relatively low cost, it will be possible to use several in parallel. One possible configuration will be 96 such devices operating in parallel, with compounds being provided from standard 96 well plates (or higher density plates), at a rate of, for example, one plate of 96 compounds every few seconds.

10 The device is also capable of immediately giving information about the potency and, optionally, the kinetics of active compounds.

The device is illustrated in the following non-limiting figures:

15 Fig.1. Shows a microfabricated conduit 1 having two input flows one of which contains the receptor 4, fluorescent ligand 5 and test compound 3. The other flow contains a liquid 2 in which the fluorescent ligand, if unbound, can diffuse into. The liquids flow out of the microcontactor area 1 into a waste unit 6. Diagram A represents the effect of fluorescent ligand diffusing across into the second flow 5 when a compound is inactive. Diagram B 20 represents the effect of non-diffusing, bound, ligand when the compound is inactive.

Fig.2. Shows an alternative arrangement from Fig.1. in which the microfabricated conduit is split into two paths the top path may be analysed directly for the presence of fluorescent ligand by a detector 7, in this diagram the compound is active. Diagram A shows the effect 25 when a compound is inactive. Diagram B shows the effect when the compound is inactive.

Fig.3. Shows an alternative arrangement where 1 is a pulsed input of compounds, 2 is the receptor, 3 is the labelled ligand, and 4 is the detector. The readout of the detector is shown at 5 where a compound which is interfering with the binding of the ligand to the receptor is 30 indicated by the broader peak compared with the neighbouring inactive compounds.

If it is considered that the test compound should interact with the ligand first, rather than the receptor, then the ligand and receptor entry ports may be reversed.

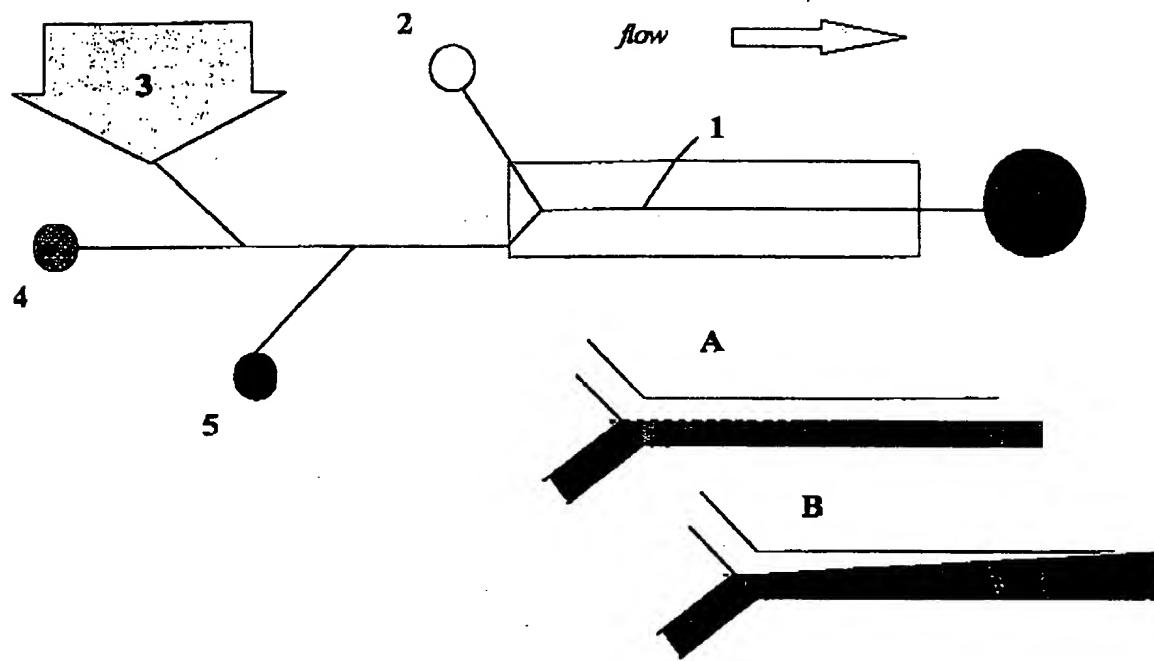
Flow may be obtained and controlled in the system by electro-osmosis using electrodes placed 5 at suitable positions on the chip, or by any other convenient means.

In a further aspect, the compound is diluted on the chip by the introduction of a buffer flow. This provides a means for continuously changing the concentration of the compounds introduced, such that a dose-response curve may be acquired and thus a measure of the 10 potency of the compound be obtained. The image analysis system may be designed to quickly identify dose response curves, even when the concentration is varied in a small time scale e.g. a few seconds or less. Optionally the chip control system could "call for" a dose response curve only when it has been determined that the compound in question has a measurable level of activity.

15

In a further aspect of the system the kinetics of binding of the compound and ligand could be determined by adjusting the flow rates and the incubation times (by for example, introducing loops that extend the length of the channels where the interactions are taking place).

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Fig.1

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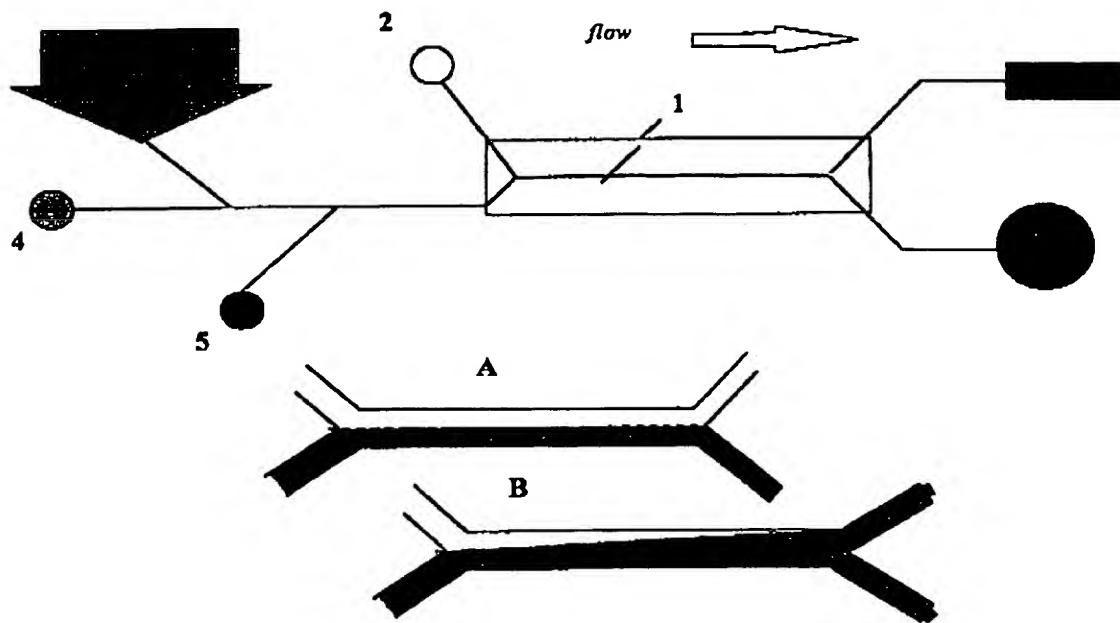


Fig.2

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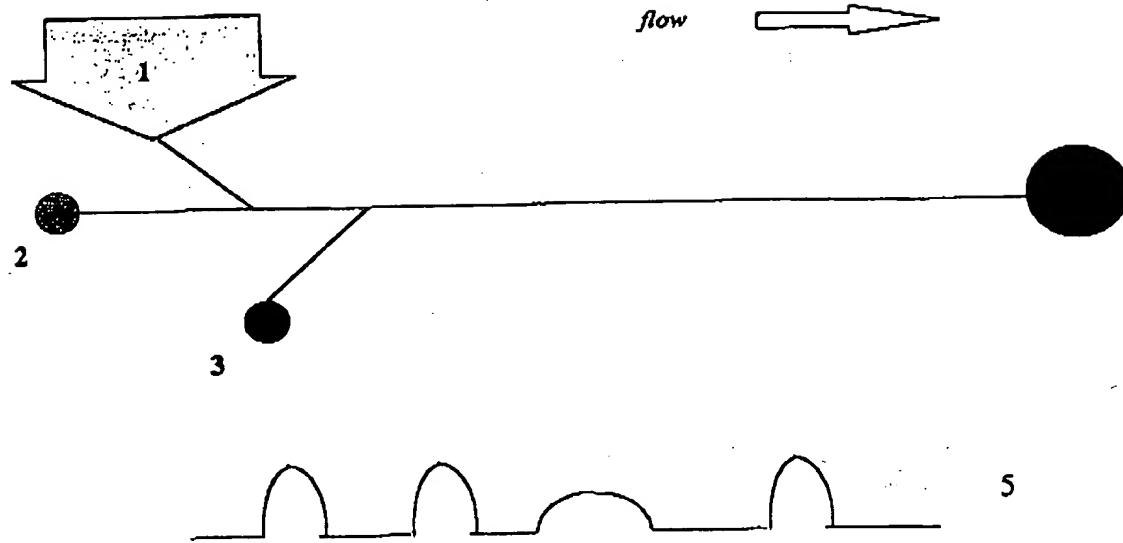


Fig.3

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